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13. ABSTRACT *(Maximum 200)*

Alteration of the HER-2/neu gene has been shown to correlate with poor prognosis in patients whose tumors contain it. This led to studies from our laboratory indicating it may play a role in the pathogenesis of the disease for some patients. Given that the HER-2/neu gene encodes a growth factor receptor found on the membrane of tumor cells and given its potential role in the pathogenesis of some human breast cancers, it is a logical target for the development of new therapeutic approaches directed at this alteration. Studies with monoclonal antibodies directed against the extra cellular domain of the receptor indicate that many may have significant growth inhibitory properties. Recently ligands have been identified which interact either directly or indirectly with the human HER-2/neu receptor, however little is known about the biologic effects of these molecules. There is some controversy as to whether the ligands mediate growth stimulatory or growth inhibitory effects or both. A greater understanding about the biologic effects of HER-2/neu overexpression as well as the impact of agonists and antagonists to the receptor has been and will continue to be required to fully therapeutically exploit this gene alteration in human breast cancer. Finally, little is know about the biologic effects of other molecular alterations which may occur in combination with HER-2/neu expression such as changes in the estrogen receptor. The proposal has been conducted to expand our knowledge base regarding the role of this critical gene in human breast cancer and to determine how to exploit this information clinically. We have completed these studies and convincingly demonstrated several aspects of the role of the HER-2 gene in human breast cancer and have designed therapeutic strategies based on the results from the models studied in this project. These studies have been taken to the clinic and some have been completed with very strong positive efficacy results.

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FINAL REPORT

DAMD17-94-J-4118

BIOLOGIC EFFECTS OF HER-2/*neu* GENE OVEREXPRESSION AND AGONISTS AND ANTAGONISTS TO THE RECEPTOR IN HUMAN BREAST CANCER

PRINCIPAL INVESTIGATOR: Dennis J. Slamon, M.D., Ph.D.

As stated in al prior progress reports on this funded proposal, the introductory section as well as the background material for the progress report for this funded proposal remain largely the same as state in the original application. Given that these sections are requested in the final report, they will again be restated here.

INTRODUCTION

The HER-2/*neu* gene is amplified in 25-30% of human breast cancers. When the alteration was examined for association with clinical parameters, it was found that gene amplification was associated with a poor prognosis; i.e. those women whose tumor contained the alteration had a shorter disease-free and overall survival. This association was initially disputed, but a large number of subsequent studies have now confirmed it. Moreover, the prognostic association between HER-2/*neu* alteration and disease outcome is seen in both node positive and node negative breast cancer. This literature and the controversy surrounding the association has been extensively reviewed recently. The bulk of the published data now clearly support the prognostic significance of HER/*neu* overexpression in breast cancer. However, all of the reviewed studies were retrospective analyses of archival cohorts of specimens. Most recently the first prospective study, consisting of 1056 primary human breast cancers, was completed and published and this study also confirmed the prognostic significance of HER-2/*neu* alteration in both node negative and node positive disease . Taken together, these data confirm the prognostic significance of HER-2/*neu* amplification/overexpression in human breast cancer.

There are at least two possible explanations for the association between HER-2/*neu* amplification/overexpression and poor clinical outcome; a) the alteration serves as a genetic marker for a poor outcome but plays no role in causing it; i.e. a useful prognostic epiphenomenon; or alternatively b) the alteration is associated with a poor outcome because it plays a direct role in the pathogenesis of such an outcome. There is circumstantial evidence which supports the latter possibility. These data include the following: 1) the mutated rat *neu* gene can act as a dominant transforming oncogene, underscoring its oncogenic potential and NIH 3T3 cells transfected with the gene are not only transformed *in vitro*, but are tumorigenic in the nude mouse ; 2) monoclonal antibodies directed against the extracellular domain of the rat *neu* gene will inhibit transformation *in vitro* as well as tumorigenicity *in vivo*; 3) studies to develop transgenic mice using the mutated rat gene under the control of an MMTV promoter, demonstrate that these mice develop breast cancer at three months of age; and finally, 5) studies using a transfected human HER-2/*neu* gene in NIH 3T3 cells demonstrate that it too will transform the cells and that higher levels of expression result in greater transforming efficiency, and greater tumorigenicity of the cells . In composite, these data clearly show the ability of the HER-2/*neu* gene to mediate transformation *in vitro* and tumorigenicity *in vivo*.

Each of these lines of circumstantial evidence, however, has shortcomings with regards to implicating the HER-2/*neu* gene in the pathogenesis of human breast cancer. The first line of evidence proved that the rat *neu* gene could be oncogenic; however, sequence analysis showed the gene to have a point mutation in the transmembrane domain. To date, no such

mutation has been identified in the human HER-2/neu gene. Instead the alteration found in human breast cancer is amplification and overexpression of the normal gene. In addition, the mutated rat neu gene induces neuroglioblastomas and not breast cancer in the animals. The monoclonal antibody studies were equally convincing that the mutated rat gene could have a role in transformation of neural tissue but, again, these antiserum were not directed against the human protein nor were they used in altering the phenotype of human breast carcinoma cells overexpressing a normal, non-mutated, human HER-2/neu gene. The transgenic mouse studies were particularly compelling in showing that alterations in the rat neu gene could result in the development of breast carcinoma but, again, the study had used the mutated rat gene (48). Lastly, the data demonstrating that an overexpressed, non-mutated human HER-2/neu gene could transform NIH 3T3 cells proved that the human gene was oncogenic in vitro, but the experiments again used NIH 3T3 cells, not human breast cancer or breast epithelial cells. In addition, the levels of overexpression in these studies were far in excess of what is seen in most human breast cancer specimens in nature. To circumvent some of the concerns raised with the experiments utilizing the rat neu gene or murine cells (NIH-3T3), we recently designed a series of experiments to introduce the human gene into human breast cancer cells as well as non-transformed, immortalized and normal non-immortalized human breast epithelial cells. Important in the studies is to mimic as closely as possible the alteration seen in human tumors and then determine the biologic effects (if any) of this alteration. Central to these studies was the use of the human gene in human cells and the objective to hold the levels of overexpression at or below those seen in actual human tumors; i.e. not to exceed levels found in primary and/or metastatic tumors in nature. These studies have not yet been published but have now been completed as part of this grant support. They are presented as preliminary data in the current report. We feel that they demonstrate our ability to conduct the studies detailed in this application.

In addition we wish to examine the effects that an additional alteration, mutation of the p53 gene, may have on cells overexpressing the HER-2/neu gene. There is considerable evidence that the conversion of a normal cell into a malignant cell is a multi step process, which may involve the alteration of more than one if not several genes. Mutation of the p53 gene is one of the most common genetic alterations found in human malignancies and is frequently found in breast cancer. The mutation rate in breast cancer is reported to be from a low of 14% to a high of 58% with the most frequently reported rate being between 25-30% of all cases. This incidence makes p53 alteration a potentially important mutation in the pathogenesis of human breast cancer. Similar to the data with HER-2/neu, this concept is circumstantially supported by data indicating that p53 mutation is associated with aggressive subtypes of the disease and/or a poor prognosis. The concept gains further support with experimental data demonstrating that introduction of a wild type p53 gene into a human breast cancer cell line containing a mutant gene will suppress the transformed phenotype. Data are now accumulating which indicate that alterations in the p53 gene are frequently associated with HER-2/neu alterations. This combination of mutations may be very important in the pathogenesis of some human breast cancers. The HER-2/neu overexpressing cell lines already developed as well as those proposed to be developed as part of this application should be useful in addressing this issue.

BODY OF REPORT

Specific Aim I - To further develop a series of human breast epithelial and cancer cell lines containing defined alterations in expression of the human HER-2/*neu* gene.

This specific aim was essentially accomplished in the first year of funding for this project, i.e. the 11/94 – 11/95 funding period. This has been detailed in the progress report of August 1997 and will be restated in brief here.

The methods used to achieve the goals of this Specific Aim involve the introduction of a full length human HER-2/*neu* c-DNA into a series of human breast epithelial cell lines representing normal breast epithelial cells, immortalized but non-transformed breast epithelial cells and breast cancer cells. The cell lines detailed in the proposal, i.e. T47D, MDA-MB-231, MDA-MB-435, BT-20 and BT-483 have all been successfully transfected and engineered to overexpress the HER-2/*neu* gene. These transfectants have been characterized for stable HER-2/*neu* overexpression and all appear to have this feature (at least at 6-months of follow-up). The biologic characterization of these cells has been similar to those studies presented in the preliminary data in the initial proposal, i.e. ^{3}H-thymidine incorporation, cell growth (*in vitro*) anchorage independent growth and tumorigenicity. In all assays the data for the newly established engineered cells are similar to the data for the MCF-7, B5 and HBL-100 cell lines. DNA synthetic rate increases significantly as does cell growth, anchorage independent growth and tumorigenicity. These data are important in that they demonstrate that the biologic effects of HER-2/*neu* overexpression seen in the MCF-7, B5 and HBL-100 cell lines are not restricted to just those cell lines but can also be achieved in all of the breast cancer cell lines evaluated. These findings lend substantial credence to the concept that overexpression of the HER-2/*neu* gene plays an important pathogenic role in the aggressive biologic behavior of those cells and tumors which contain it. More recent studies using some of these cell lines has lead to insights into the clinical observation that human breast cancers which overexpress the HER-2/*neu* receptor tend to be estrogen receptor negative. Studies performed in our laboratory and, in part, supported by the grant, have demonstrated a potentially important direct interaction between activation of the HER-2/*neu* receptor and down regulation of the estrogen receptor. This is a critical area of research currently as there are developing data indicating a relationship between HER-2/*neu* overexpression and estrogen receptor negativity and tamoxifen resistance. This research has been expanded as part of this proposal in Specific Aim 4. We have also used these cell lines to study the effects of HER-2/*neu* overexpression on expression of molecules believed to be involved in adhesion of cells to other cells as well as stroma and matrix elements in various tissues. The rationale behind this approach is to evaluate the potential mechanism for the observation that HER-2/*neu* over-expressing cells have an increased metastatic potential in vivo models. Using the transfected HBL-100 cells for which we have a number of clones which range in HER-2/*neu* expression from normal levels to levels of overexpression seen in breast cancer specimens with amplification of the gene, we have determined that there are definite changes which occur in ß-catenin, E-cadherin, ICAM-1, integrins -$\alpha\beta$ 5, $\beta\alpha$ 6 and B1 as well as FAK, depending on HER-2 levels.

The most recent data generated with these cell lines bear directly on a critical area of the aggressive clinical behavior of HER-2/*neu* overexpressing breast cancer cells. The new data, generated since the last progress report is that HER-2/*neu* overexpression is consistently associated with overexpression of the angiogenic molecule, vascular endothelial growth factor (VEGF). The significant increase in VEGF expression in all of the cell lines engineered to overexpress HER-2/*neu* (as compared to their parental counterparts) is consistent with the increase in metastatic potential seen in HER-2/*neu* positive tumors. This phenomenon affords an additional therapeutic approach, which would be rational in these tumors, i.e. inhibition of VEGF activity by use of antagonists to this growth factor. As a direct result of the most recent data generated in this specific aim, we will test this approach in preclinical studies in future experimentation on this system. In addition, we will utilize these cell lines for analysis in

expression chip assay technology approaches which we hope will give some insights into other genes differentially expressed in HER-2/*neu* overexpressing versus non-HER-2/*neu* overexpressing cells. These studies could lead to the identification of other molecules, which may serve as targets in HER-2/*neu* positive human breast cancers.

Specific Aim II - To assess the biologic effects of agonists, i.e. the heregulin and *neu* differentiation factor ligands as well as an antagonist, i.e. a monoclonal antibody, to the HER-2/*neu* reception on human breast cells, *in vitro.*

These studies have lead ultimately to the clinical testing of the anti-HER-2/*neu* monoclonal antibody (Herceptin) in tumors with HER-2/*neu* positive breast cancers. Almost all of this work was completed by the time of the last progress report, August 1997 and have been summarized in that report. In brief review, a ligand which activates the HER-2/*neu* receptor, i.e. heregulin, was tested in our cell lines developed in specific aim I as well as the available naturally HER-2/*neu* overexpressing cell line SKBR2. The data from these studies show a consistent growth stimulatory pattern of the agonist heregulin in these cells both in vitro and in vivo. Rather than restate all of the data in graph and figure form here, we have enclosed a preprint of a manuscript entitled "Biologic effects of heregulin/*neu* differentiation factor on normal and malignant human breast and ovarian epithelial cells." Buy Aguilar, et al. This work has recently been accepted for publication in <u>Oncogene</u> and details all of this data achieved on our work with agonists.

The most recent effects of antagonists, i.e. the monoclonal antibody, Herceptin, has also been accepted for publication and the data for this work is enclosed in the preprint of a second manuscript by Pegram et al., entitled "Inhibitory effects of combinations of HER-2/*neu* antibody and chemotherapeutic agents used for treatment of human breast cancer". This work has also been recently accepted for publication in <u>Oncogene</u> and has served and we believe, will continue to serve as a source for testing of combinations of anti-HER-2/*neu* monoclonal antibodies and chemotherapeutic reagents.

Specific Aim III - To assess the biologic effects of ligand and antibody, alone and in combination, on HER-2/*neui* expressing human breast cancer cells in vivo.

The most recent work completed on this specific aim has been on the effects of anti-HER-2/*neu* antibody and radiation therapy. This work was alluded to in the last progress report and the preliminary data was shown in graph and figure form in that report. Since that report, this work has been completed and was submitted to and accepted for publication in <u>Cancer Research</u>. This paper entitled: Monoclonal antibody to HER-2/*neu* receptor modulates repair of radiation-induced DNA damage and enhances radiosensitivity of human breast cancer cells overexpressing this oncogene", by Pietras et al, is included with this report demonstrating the most recent interaction found between antibodies to the HER-2/*neu* receptor and therapeutic modalities.

Specific Aim IV - To assess the biologic effects of an additional mutation of the p53 gene in combination with HER-2/*neu* overexpression in human breast cells both in vitro and in vivo.

As stated in the past two year's progress reports we have not been able to successfully develop stable transfectants containing both HER-2/*neu* overexpression and p53 mutant gene expression. All attempts at developing such stable transfectants have failed. Two years ago we changed the main objective of this specific aim to address the association between HER-2/*neu* overexpression and hormone dependence and tamoxifen resistance in breast cancer again with the objective of better understanding and defining the interaction between the HER-2/*neu* genetic alteration and the functional biology of the estrogen receptor. The data presented in the past progress report demonstrated that HER-2/*neu* overexpression was associated with downregulation of the ER. The preclinical studies were restricted to observations in a single cell

line, MCF-7 cells. We have consistently attempted to demonstrate biologic phenomenon associated with HER-2/*neu* overexpression is in more than one cell line to be certain that the observed effects were not restricted to a single cell line. To this end we have undertaken transfection studies with the ZR 75 human breast cancer cell line. These cells do not overexpress HER-2/*neu* and using the same transfection strategy they were successfully engineered to overexpress HER-2/*neu* at levels seen in pathologic human material (Fig 1). Moreover, once they do overexpress HER-2/neu, they become resistant to tamoxifen treatment (Fig. 2). These data together with very similar data on MCF 7 cells (see last progress report) provide compelling evidence that HER-2/neu overexpression is directly linked with an ER negative estrogen independent, tamoxifen resistant phenotype. This information could prove critically important in treatment decisions not only for hormonal therapy but also for strategies of adding Herceptin to standard hormonal manipulation. This will be an active area of study by us as well as by a number of other groups.

CONCLUSION

Overall this project was designed to address fundamental questions regarding the biologic effects of HER-2/*neu* expression in human breast cancer. The objectives were to design and develop a series of human breast cancer cell lines which could then be used in in vitro and in vivo studies to assess effects of various critical molecules on this altered growth regulatory pathway. The conclusions of the project demonstrate that HER-2/*neu* overexpression consistently confers aggressive biologic behavior to those human breast cancer cells in which it is overexpressed at levels seen in pathologic materials, i.e. breast cancers containing this alteration. These changes include increased DNA synthesis rates, increased in vitro growth rates (both anchorage dependent and anchorage independent growths) and increased tumorgenicity in vivo. The studies went on to demonstrate that agonists to this receptor consistently generate a growth stimulatory response when used in HER-2/*neu* overexpressing cells. Moreover, the studies went on to show antibodies directed against the entire cellular domain of this receptor could have profound growth inhibitory effects on cells overexpressing the receptor while having no effects on cells which contain normal levels of HER-2/*neu*. In addition, further studies demonstrated while the antibodies themselves had growth inhibitory effects, combinations of the antibodies and other traditional therapeutic interventions, i.e. chemotherapeutic drugs and ionizing radiation, could provide additive and sometimes synergistic therapeutic effects. We were also able to demonstrate using the reagents (cell lines and animal models) generated from the project, that HER-2/*neu* overexpression alone does not confer intrinsic drug sensitivity or drug resistance to breast cancer cells which contain this change. Finally, we have been able to demonstrate that HER-2/*neu* overexpression is directly associated with development of an estrogen independent, estrogen receptor negative, tamoxifen resistant phenotype.

Taken together, these data and the publications resulting from this work provide convincing evidence that HER-2/*neu* overexpression is associated with a poor prognosis because this alteration plays a direct role in the pathogenesis of these breast cancers in which it occurs. In addition we have successfully demonstrated through these studies that this alteration can be successfully targeted with antibodies directed against the receptor and have developed further strategies using combinations of the antibodies and traditional therapeutic agents. Finally we have demonstrated that HER-2/*neu* overexpression plays a potentially central role in the development of hormone resistance in human breast cancer and have developed strategies on how this might be successfully reversed.

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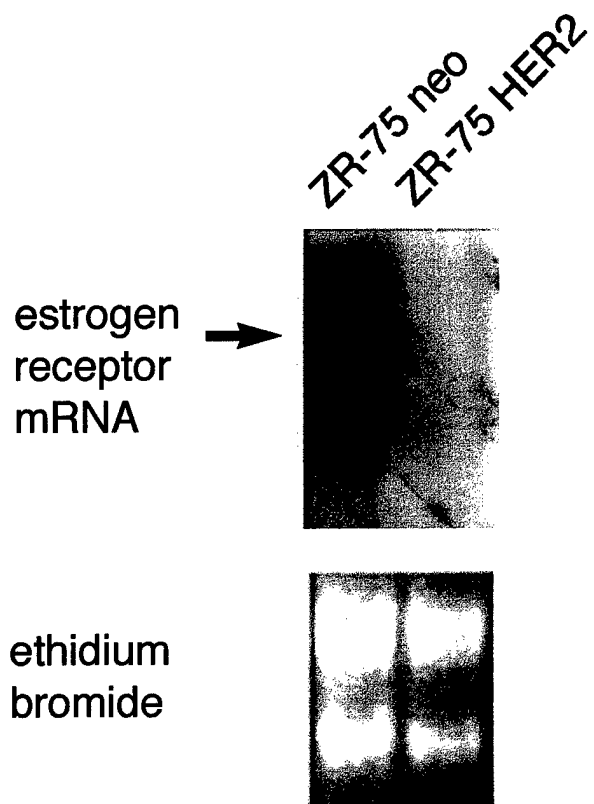Natarajan Venkatesan

estrogen
receptor
mRNA

ethidium
bromide

Figure 1. The estrogen receptor message is down regulated in ZR-75
breast cancer cells overexpressing HER-2. The top panel represents a
Northern blot of total RNA extracted from ZR-75 breast cancer cells
transfected with a control vector (neo) or a vector containing the HER-2
coding region. The first 1.3 kb of the estrogen receptor coding region
was used as the probe. The lower panel shows the ethidium bromide
staining of the total RNA prior to transfer as loading control.
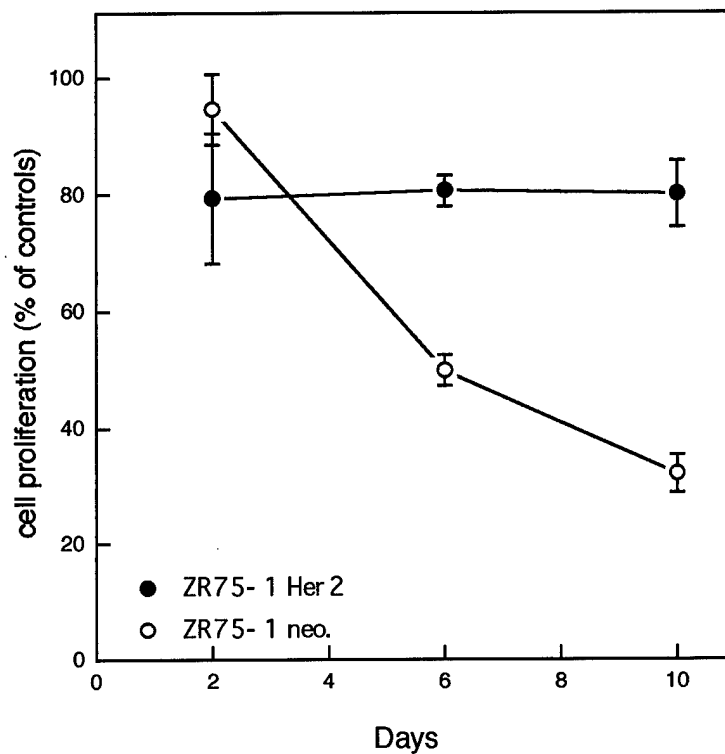
Figure 2. HER-2 overexpression confers tamoxifen resistance in ZR-75 breast cancer cells. ZR-75 cells having stable expression of the neomycin gene from a control vector (neo) or the coding sequence of HER-2 were grown in 24 well plates in RPMI+10% FBS. After plating, cells were treated with10 nM 4-hydroxy-tamoxifen (4-OH-TAM) or solvent alone (ETOH). Triplicate wells were harvested and counted on days 2,6 and 10. The data and standard deviationis are plotted as the perentage of cells in the 4-OH-TAM treated wells relative to solvent treated control wells.

Monoclonal Antibody to HER-2/*neu* Receptor Modulates Repair of Radiation-induced DNA Damage and Enhances Radiosensitivity of Human Breast Cancer Cells Overexpressing This Oncogene[1]

Richard J. Pietras, Joseph C. Poen, David Gallardo, P. Nancy Wongvipat, H. Julie Lee, and Dennis J. Slamon[2]

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ABSTRACT

The management of human breast cancer frequently includes radiation therapy as an important intervention, and improvement in the clinical efficacy of radiation is desirable. Overexpression of the HER-2 growth factor receptor occurs in 25–30% of human breast cancers and correlates with poor clinical outcome, including earlier local relapse following conservative surgery accompanied by radiation therapy. In breast cancer cells with overexpression of HER-2 receptor, recombinant humanized monoclonal antibodies (rhuMAbs) to HER-2 receptors (rhuMAb HER-2) decrease cell proliferation *in vitro* and reduce tumor formation in nude mice. Therapy with rhuMAb HER-2 enhances tumor sensitivity to radiation at doses of 1–5 Gy, exceeding remission rates obtained with radiation alone. This benefit is specific to cells with HER-2 overexpression and does not occur in cells without overexpression. Treatment of cells with radiation (2–4 Gy) alone provokes a marked increase in unscheduled DNA synthesis, a measure of DNA repair, but HER-2-overexpressing cells treated with a combination of rhuMAb HER-2 and radiation demonstrate a decrease of unscheduled DNA synthesis to 25–44% of controls. Using an alternate test of DNA repair, *i.e.*, radiation-damaged or undamaged reporter DNA, we introduced a cytomegalovirus-driven β-galactosidase into HER-2-overexpressing breast cancer cells that had been treated with rhuMAb HER-2 or control. At 24 h posttransfection, the extent of repair assayed by measuring reporter DNA expression was high after exposure to radiation alone but significantly lower in cells treated with combined radiation and rhuMAb HER-2 therapy. To further characterize effects of rhuMAb HER-2 and the combination of antibody and radiation on cell growth, analyses of cell cycle phase distribution were performed. Antibody reduces the fraction of HER-2-overexpressing breast cancer cells in S phase at 24 and 48 h. Radiation treatment is also known to promote cell cycle arrest, predominantly at G_1, with low S-phase fraction at 24 and 48 h. In the presence of rhuMAb HER-2, radiation elicits a similar reduction in S phase at 24 h, but a significant reversal of this arrest appears to begin 48 h postradiation exposure. The level of S-phase fraction at 48 h is significantly greater than that found at 24 h with the combined antibody-radiation therapy, suggesting that early escape from cell cycle arrest in the presence of antireceptor antibody may not allow sufficient time for completion of DNA repair in HER-2-overexpressing cells. Because it is well known that failure of adequate p21WAF1 induction after DNA damage is associated with failure of cell cycle arrest, we also assessed the activity of this critical mediator of the cellular response to DNA damage. The results show induction of p21WAF1 transcripts and protein product at 6, 12, and 24 h after radiation treatment; however, increased levels of p21WAF1 transcript and protein are not sustained in HER-2-overexpressing cells exposed to radiation in the presence of rhuMAb HER-2. Although transcript and protein levels increase at 6–12 h, they are both diminished by 24 h. Levels of p21WAF1 transcript and protein at 24 h are significantly lower than in cells treated by radiation without antibody. A reduction in the basal level of p21WAF1 transcript also occurred after 12–24 h exposure to antibody alone. The effect of HER-2 antibody may be related to tyrosine phosphorylation of p21WAF1 protein. Tyrosine phosphorylation of p21WAF1 is increased after treatment with radiation alone, but phosphorylation is blocked by combined treatment with antireceptor antibody and radiation. This dysregulation of p21WAF1 in HER-2-overexpressing breast cells after treatment with rhuMAb HER-2 and radiation appears to be independent of p53 expression levels but does correlate with reduced levels of mdm2 protein. These data indicate that human breast cancer cells damaged by radiation may be especially vulnerable to injury if they are also deprived of essential signal transduction pathways provided by the HER-2 growth factor receptor pathway.

INTRODUCTION

In patients with breast cancer, adjuvant radiation therapy is an important therapeutic intervention following breast conservation surgery (1, 2). Radiation therapy is also recommended after total mastectomy and lymph node dissection for women with large primary cancers or extensive lymph node metastasis. Despite these interventions, however, local recurrence still accounts for an estimated 30–50% of all first recurrences in subgroups of patients (2). Given these data, it is clear that improvement in the efficacy of adjuvant radiation therapy is desirable. The response of breast malignancies to ionizing radiation is commonly the result of DNA injury. Human cells exhibit complex responses to DNA damage, including activation of genes involved in cell cycle arrest, DNA repair, and apoptosis. Recent findings suggest that the cellular response to DNA damage is markedly impaired by deprivation of essential growth factors or by blockade of growth factor receptors (2, 3). Signal transduction pathways mediated by receptor tyrosine kinases and protein kinase C appear to be important for the induction of many of the genes related to key cellular functions that permit the cell to survive a dose of radiation. Specific blockade of these pathways in tumor cells may provide attractive targets for increasing the cytotoxic effects of radiation. Growth factors and their receptors also play a pivotal role in the regulation of human breast cell growth and differentiation. Among growth factor receptors, the most frequently implicated in the pathogenesis of human breast cancer have been members of the erb B receptor family, especially the HER-2 (erb B2) protein, a M_r 185,000 transmembrane receptor tyrosine kinase encoded by HER-2/*neu* proto-oncogene (4). HER-2 is amplified and overexpressed in 25–30% of human breast cancers (5–8), and overexpression of the structurally unaltered *HER-2* gene leads to neoplastic transformation of NIH-3T3 cells (9–11) and immortalized human breast cells (8, 12), indicating that this alteration plays a pathogenic role in promoting tumorigenicity of nonmalignant cells. Monoclonal antibodies against the extracellular domain of HER-2 specifically inhibit the growth of human breast carcinoma cells overexpressing the HER-2 gene product (8). Amplification of the *HER-2* oncogene has also been shown to correlate with distant relapse and overall patient survival in patients with breast cancer (5–8, 13–15). More recently, overexpression of

HER-2 has been found to correlate with the risk for local relapse in patients treated only with conservative surgery and radiation (16).

A potential correlation between HER-2 receptor overexpression and sensitivity to DNA damage from chemotherapeutic drugs and radiation, derived from several laboratory studies, may prove to be clinically useful. The initial studies implicating an interaction between inhibitors of type I receptor tyrosine kinases by antibodies and response to DNA-damaging agents were part of the studies of the EGF[3] receptor. These studies indicated a synergistic effect between antibodies to EGF receptor and the chemotherapeutic drug, cisplatin (17). This combined treatment elicited a significant reduction in both the number and size of epidermoid cancers grown as s.c. xenografts in athymic mice (see also Ref. 18). EGF is also reported to enhance the sensitivity of human squamous carcinoma cells to radiation therapy (19). The potential role of proto-oncogenes for EGF and HER-2 receptors in the modulation of sensitivity to radiation has also been suggested from limited laboratory studies (20, 21). We have shown that blockade of the HER-2 receptor in human breast cancer cells using an anti-HER-2 antibody promotes a synergistic antitumor effect when combined with the DNA-damaging drug, cisplatin (22), and that the signal generated by antibody binding to HER-2 receptors may block DNA repair in HER-2-overexpressing cells (23). Nerve growth factor is also known to reduce DNA repair induced by UV radiation and to slow removal of DNA adducts induced by benzo(a)pyrene in neuroblastoma cells (24). However, the specific molecular pathways used by cells for suppression of DNA repair, which are triggered by ligand (or antireceptor antibody) interactions, remain unclear. The tumor suppressor gene product p53 is a critical mediator of the cellular response to DNA damage. Both cell cycle arrest (25) and programmed cell death (26) after DNA damage due to ionizing radiation are closely linked to p53 function. Recent data further suggest that p53 may be critical in the repair of DNA damage (27). Many of the effects of p53 can be attributed to the function of downstream p53-regulated genes, including *p21WAF1*, also known as Cip1, sdi1, or CAP20, that codes for a M_r 21,000 protein (p21WAF1; Ref. 28). Cell cycle checkpoints on progression of cells through the G_1 and into S phase are controlled by protein kinases, the CDKs, as well as their activating partners, the cyclins. Binding of cyclin/CDK complexes by p21WAF1 leads to inhibition of cyclin/CDK and results in cell cycle arrest (28, 29). Many of these responses to DNA damage are regulated, in turn, by growth factor signaling pathways (3, 30). Recent reports show p53-independent activation of p21WAF1 by MAP kinase signaling (31). In addition, withdrawal of growth factors *in vitro* has been associated with down-regulation of p21WAF1 expression and enhanced cell killing in response to DNA damage (3).

Here, we tested the hypothesis that DNA damage induced by external-beam radiation can be combined with agents designed to act selectively against cells in which the HER-2 receptor pathway is altered for therapeutic advantage. Cross-communication between DNA damage response pathways involving p21WAF1 and growth factor signaling pathways, *i.e.*, HER-2, is evaluated in breast cancer cells with HER-2 overexpression.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The human breast carcinoma cell line, SKBR3, constitutively overexpresses the *HER-2* gene and was obtained from the American Type Culture Collection (Manassas, VA). Stable retroviral transfectants of MCF-7 human breast cancer cells that overexpress the HER-2

receptor (MCF-7/HER-2) have been prepared (11, 22). Cells infected with a control retroviral vector not containing the *HER-2* gene (MCF-7/CON) as well as parental cells not infected with retrovirus (MCF-7/PAR) were used as controls in *in vitro* and *in vivo* experiments. All cells were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM freshly added glutamine, and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA).

Anti-HER-2 Receptor Monoclonal Antibodies. rhuMAb to HER-2 receptor (rhuMAb HER-2; generously provided by Genentech, South San Francisco, CA), derived from the murine monoclonal antibody 4D5, is directed to an extracellular epitope of HER-2 receptor. Antibodies were prepared as described in detail elsewhere (32, 33). Control experiments were conducted with nonspecific IgG of the same class and isotype to verify the specificity of any observed effects.

Tumor Formation in Nude Mice. Human breast cancer cells were inoculated s.c. at 5×10^7 cells/animal in the hind thighs of 3-month-old female athymic mice. Prior to inoculation, mice were primed for 10–14 days with 17β-estradiol applied s.c. in a biodegradable carrier-binder (1.7 mg of estradiol per pellet) to promote growth of these estrogen-dependent breast cancer cells. Tumor nodules were monitored by dimension measurements (in mm). Five to six animals were included in each treatment group, with randomization by body weight and tumor nodule size at the start of each experiment. Antibody treatment was initiated when tumors grew to ≥50 mm³ in size in one set of experiments or to >350 mm³ in size in independent studies. Monoclonal antibody and control solutions were administered by i.p. injection. The rhuMAb HER-2 antibody was given at a dose of 5 or 10 mg/kg animal weight in three doses at 4-day intervals (over 12 days). Control injections included human IgG1 (5 or 10 mg/kg), given on a similar treatment protocol. Radiation treatments were designed to mimic protocols in use in the clinic, and radiation to tumors growing s.c. on a peripheral rear extremity was performed with special planning to avoid inappropriate radiation exposure to non-tumor-bearing sites. Mice were sacrificed for pathological examination under supervision of the institutional veterinarian. Euthanasia technique was cervical dislocation, which is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Tumors were analyzed to confirm HER-2 expression by immunohistochemistry as described elsewhere (6).

Measurement of Cell Cycle Phase Distribution. MCF-7 cells with HER-2 overexpression were plated on plastic at low density and allowed to adhere. After 48 h, the monolayers were washed with PBS and incubated in medium with 1% DCC-stripped FCS to arrest cell growth. After 48 h, this medium was replaced with medium containing either 1% DCC-treated FCS or 10% FCS with or without 200 μg/ml monoclonal antibody to the HER-2 receptor. Cells in medium with 10% FCS but without antibody were treated with 4 Gy of radiation, and cells with 10% FCS plus 200 μg/ml antibody were similarly treated. Cells from all treatment groups were incubated further for 24 or 48 h. Cells were then prepared for cell cycle analysis by established methods, using DNA staining with propidium iodide and flow cytometry (34). The proportion of cells in S phase was quantitated to assess effects of DNA damage and modulation by the antibody (34).

Measurement of DNA Repair. UDS, a type of DNA repair that is non-semiconservative in nature, is a well-established measure of the effects of ionizing radiation (22, 35). Using methods detailed previously (22), we measured UDS by autoradiographic approaches (36) in parent and daughter cells with and without exposure to antireceptor antibody and radiation, either given alone or in combination. Treatment with antibody (200 μg/ml) occurred for 4 h before exposure to radiation.

An alternate measure of DNA repair involved introduction of radiation-damaged or reporter DNA into breast tumor cells. Prior to transfection, a reporter DNA, CMV-driven β-galactosidase (pCMV-β; Clontech), was prepared with or without exposure to ionizing radiation *in vitro*, using methods reported previously (37). For transfection experiments, cells were plated 24 h prior to transfection, and transfections with internal controls to measure transfection efficiency were carried out as described previously (37, 38). Undamaged or radiation-damaged DNA (1.5 μg) was used in these transfection experiments. At 24 h posttransfection, the extent of repair was assayed by measuring reporter DNA expression. The transfected cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a substrate for β-galactosidase, to distinguish between β-galactosidase-positive and -negative cells. In

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[3] The abbreviations used are: EGF, epidermal growth factor; CDK, cyclin-dependent kinase; MAP kinase, mitogen-activated protein kinase; rhuMAb, recombinant humanized monoclonal antibody; DCC, dextran-coated charcoal; UDS, unscheduled DNA synthesis; CMV, cytomegalovirus; PCNA, proliferating cell nuclear antigen.

the presence of substrate, cells expressing bacterial β-galactosidase appeared blue, and the percentage of stained cells was quantitated.

Measurement of p21WAF1 Transcript and Protein Levels. Transcripts of p21WAF1 were determined by Northern blot analysis, using established protocols (5, 6, 28, 38). In brief, breast cancer cells with and without HER-2 overexpression were treated with or without rhuMAb HER-2 for 4–24 h before exposure to radiation (0–20 Gy). Cells were then maintained for 6, 12, or 24 h prior to harvesting and processing for collection of RNA. After Northern blot analysis, the resulting blots were hybridized with p21WAF1 cDNA (2.1 kb, *Not*I; Ref. 28). In control studies, some blots were hybridized with cDNA for human p53 (2.0-kb *Bam*HI fragment) or human cyclin D (American Type Culture Collection). These probes were ^{32}P-labeled by a random priming method (39).

Western analyses of the level of p21WAF1 protein in breast cancer cells were conducted with methods described previously (38). We assessed p21WAF1 protein in response to DNA damage in breast cancer cells in the presence and absence of growth factor receptor antibody. Breast cancer cells with and without HER-2 overexpression were treated with 100 μg/ml rhuMAb HER-2 for 4–24 h before exposure radiation. Cells were then maintained for 6 and 24 h prior to harvesting and processing of cell lysates for electrophoresis (38). Immunoblotting was performed with MAb 6B6 with specificity for human p21WAF1 (PharMingen). In other control studies, immunoblotting was also performed with monoclonal antibodies to PCNA (Santa Cruz Biotechnology), p53 (PAb1801, AB-2; Oncogene Science), mdm2 (Oncogene Research Products), and bcl-2 (Transduction Laboratories).

RESULTS

Sensitivity of Breast Cancer Cells with HER-2 Overexpression to Radiation and Effects of Anti-HER-2 Antibody. We have directly compared the *in vitro* radiation sensitivities of parental breast cancer cells with normal expression levels of HER-2 to that of matched daughter cells containing HER-2 overexpression (Fig. 1A). Survival curves were obtained after treatment of cells with graded doses of ionizing radiation at a dose rate of 1 Gy/min with doses of 0, 1, 2, 4, and 5 Gy. After the radiation treatment, cells were placed into 35-mm dishes and cultured for 14 days, with the surviving fractions quantitated at day 14 (20). Irradiation of MCF-7/HER-2 cells *in vitro* resulted in a D_{10} (the dose required to reduce cell survival to 10%) that was increased by ~25% relative to MCF-7/control cells (Fig. 1A). These analyses suggest a potential biological role of the *HER-2* oncogene in resistance to radiation treatment. Alternatively, this apparent difference in sensitivity could be the result of a differential growth rate because HER-2-overexpressing cells have been found to exhibit more rapid regrowth than control cells following the initial response to chemotherapy (40).

To evaluate the *in vitro* effect of radiation combined with antibodies to HER-2, we conducted studies using the transfected MCF-7 cells (Fig. 1A) as well as the naturally HER-2-overexpressing SKBR3 breast cancer cells (Fig. 1B). Cells cultivated either on plastic (MCF-7/HER-2 cells) or in soft agar plates (SKBR3 cells) with or without rhuMAb HER-2 were treated with or without radiation prior to incubation *in vitro* (see Fig. 1). These data show that anti-HER-2 receptor antibody enhances radiation-induced killing of human breast cancer cells with HER-2 overexpression.

***In Vivo* Effects of Ionizing Radiation Combined with Anti-HER-2 Antibody on Human Breast Cancer Xenografts.** To evaluate the efficacy of radiation therapy with rhuMAb HER-2 on the growth of MCF-7/HER-2 xenografts, we inoculated cells into estrogen-primed female athymic mice for 14 days and allowed them to grow to 50–100 mm³ prior to randomization into four groups. Treatment groups included control human IgG1 at 30 mg/kg (Fig. 2A, *CON*), radiation at 4 Gy with human IgG1 (*RT*), rhuMAb HER-2 at 30 mg/kg (*MAb*), or combined radiation/rhuMAb (*RT/MAb*) therapy (Fig. 2A). Doses of antibody or IgG1 were administered in divided doses on
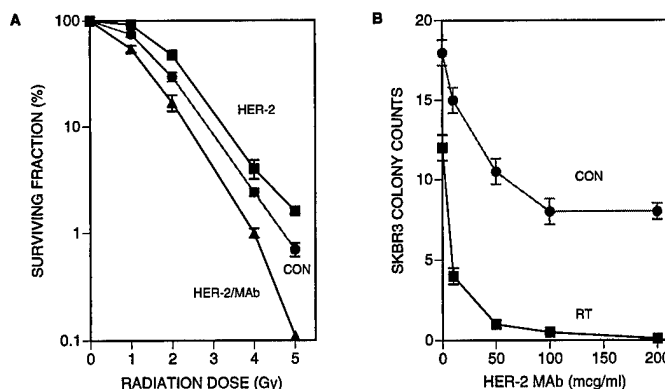

Fig. 1. Human breast cancer cells with overexpression of HER-2 growth factor receptor show radiation resistance that can be reversed by treatment with antibody to HER-2 receptors. *A*, survival curves were obtained for cells after treatment with graded doses of ionizing radiation at a dose rate of 1 Gy/min with doses of 0, 1, 2, 4, and 5 Gy. The treatment groups included MCF-7 control breast cancer cells with low-expression of HER-2 (*CON*, ●), HER-2-overexpressing MCF-7 breast cancer cells (*HER-2*, ■), and HER-2-overexpressing MCF-7 cells treated with rhuMAb to HER-2 receptor at 200 μg/ml beginning 2 h prior to radiation (*HER-2/MAb*, ▲). After radiation treatment, cells were divided into 35-mm dishes and cultured over 14 days. *Data points*, means from three experiments; *bars*, SE. Effects of *HER-2* gene overexpression on increased cell survival are significantly different from control ($P < 0.05$), and treatment with antibody to HER-2 receptor is associated with a reversal of this radioresistance ($P < 0.05$). D_0, the final slope of the radiation dose-cell survival curve, is 1.03 ± 0.14 for HER-2-overexpressing cells, 0.79 ± 0.06 for control cells, and 0.44 ± 0.17 for HER-2-overexpressing cells treated with HER-2 antibody, with the latter D_0 significantly different from that of both HER-2-overexpressing and control cells ($P < 0.001$). *B*, human breast cancer cells with natural overexpression of HER-2 receptor, SKBR3, were treated *in vitro* with rhuMAb HER-2 at 0–200 μg/ml, without radiation (*CON*) or with adjuvant radiation treatment at 2 Gy (*RT*). To study the effect of rhuMAb HER-2 on anchorage-independent growth, we plated cells at a density of 2.5 × 10⁴ cells per 6-cm dish in triplicate. The dishes consist of 0.4% (w/v) agar bottom layers and a 0.2% (w/v) agar top layers. Cells were plated in between the latter two layers with rhuMAb HER-2 or control solution. Plates were irradiated at 2 Gy or maintained as nonirradiated controls and then incubated at 37°C/5% CO₂ for 3 weeks, at which time colonies were counted. Treatment with antibody to HER-2 receptor in combination with radiation elicits reduced colony formation in agar compared to the effect of antibody therapy alone ($P < 0.01$).

days 1, 4, and 7. Those groups treated with radiation received a treatment 4 h after administration of antibody or control IgG1 on days 1, 4, and 7. Tumor nodules were monitored through day 49. The effect of repeated doses of rhuMAb HER-2 with or without ionizing radiation on tumor volume in the various groups was measured (Fig. 2A). In mice receiving low doses of radiation with control IgG (Fig. 2A, *RT*), mean tumor volumes were not significantly reduced over the 7-week observation period, and no tumor remissions were observed compared to controls (*CON*). Tumors treated with rhuMAb HER-2 alone (*MAb*) also failed to show significant growth reduction compared to controls and, again, no tumor remissions were noted. In contrast, combined radiation-antibody therapy produced marked reduction in tumor volumes over the 7-week treatment period compared to control or either treatment alone ($P < 0.001$). Moreover, all animals that received both rhuMAb HER-2 and radiation (Fig. 2A, *RT/MAb*) had complete tumor remissions.

To better evaluate the *in vivo* efficacy of radiation therapy with rhuMAb HER-2 on the growth of larger MCF-7/HER-2 xenografts, cells were inoculated in estrogen-primed female athymic mice and allowed to form 350–400-mm³ tumors prior to randomization into four groups. Treatment groups included control human IgG1 at 15 mg/kg (Fig. 2B, *CON*), radiation at 8 Gy with human IgG1 (*RT*), rhuMAb HER-2 at 15 mg/kg (*MAb*), and combined radiation/rhuMAb (*RT/MAb*) therapy. Doses of antibody or IgG1 were administered in divided doses on days 1, 4, and 7. Those groups treated with radiation received a treatment 4 h after antibody or control IgG1 only on day 1. Tumor nodules were monitored through day 15. A lower dose of rhuMAb HER-2 with or without ionizing radiation was used, and
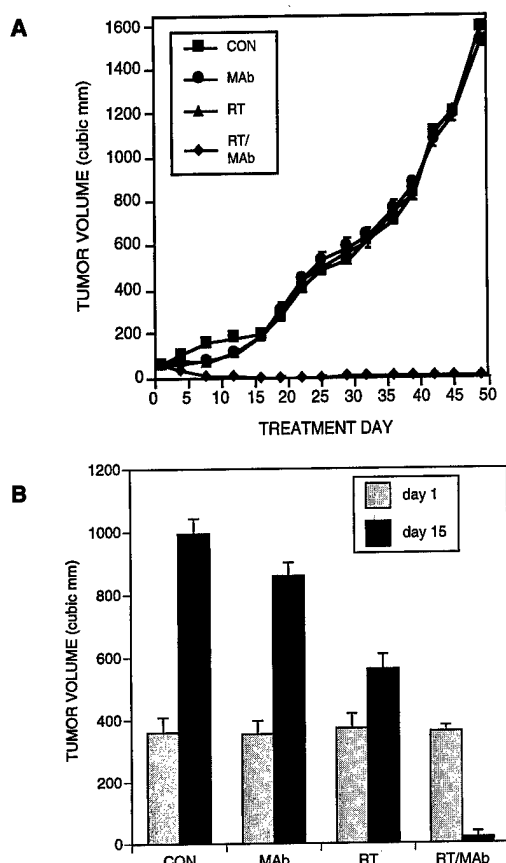
Fig. 2. Combination treatment with antibody to HER-2 growth factor receptor and radiation promotes remission of human breast cancer xenografts in nude mice. *A*, MCF-7/HER-2 cells were injected s.c. at 5×10^7 cells per nude mouse. After 14 days, mice were randomized on day 0 to groups of six animals on the basis of body weight and tumor nodule size. Treatment groups included human IgG1 control at 30 mg/kg (*CON*, ■), radiation at 4 Gy with human IgG1 (*RT*, ▲), rhuMAb HER-2 at 30 mg/kg (*MAb*, ●), or combined radiation/rhuMAb (RT/MAb, ◆) therapy. Doses of antibody or IgG1 indicated above were administered in divided doses on days 1, 4, and 7. Those groups treated with radiation received a 4-Gy treatment at 4 h after administration of antibody or IgG1 on days 1, 4, and 7 only. Tumor nodules were monitored to day 49. On postmortem examination, no residual tumor cells were found by light microscopy in the RT/MAb treatment group, but cancer cells were found in the s.c. nodules of mice from all other treatment groups. *B*, MCF-7/HER-2 cells were injected s.c. at 5×10^7 cells per nude mouse. After 35 days, mice were randomized on day 0 to groups of three to five animals on the basis of body weight and tumor nodule size, with tumors ranging in size from 350 to 400 mm³. Treatment groups included human IgG1 control at 15 mg/kg (*CON*), radiation at 8 Gy with human IgG1 (*RT*), rhuMAb HER-2 at 15 mg/kg (*MAb*), or combined radiation/rhuMAb (*RT/MAb*) therapy. Doses of antibody or IgG1 indicated above were administered in divided doses on days 1, 4, and 7. Those groups treated with radiation received an 8-Gy treatment at 4 h after administration of antibody or IgG1 on day 1 only. Tumor volumes were recorded at day 1 and reassessed at 15 days.

effects on tumor volume in the various groups were measured (Fig. 2*B*). In mice receiving one administration of radiation at 8 Gy with control IgG (*RT*), mean tumor volumes were reduced at 15 days when compared to controls (*CON*; $P < 0.05$). Tumors treated with rhuMAb HER-2 alone (*MAb*) showed less growth reduction, but combined radiation-antibody therapy produced marked reduction in tumor volumes over the 15-day treatment period when compared to control or either treatment alone ($P < 0.001$). These data, in combination with the results in Fig. 2*A*, show superior efficacy of radiation when given with rhuMAb HER-2 and demonstrate a clear therapeutic advantage with this treatment regimen.

Anti-HER-2 Antibodies Block DNA Repair in Response to Ionizing Radiation in Human Breast Cancer Cells. After demonstrating a therapeutic advantage for the combination of antibody and radiation in HER 2-overexpressing cells, studies were designed to evaluate the possible mechanism(s) for this phenomenon. Studies

have shown that DNA repair plays an important role in the recovery of cells from the toxicity of ionizing radiation (41). Prior work has also shown that inhibition of DNA repair by anti-HER-2 receptor antibodies is important in antibody-enhanced cytotoxicity of cisplatin in HER-2-overexpressing breast and ovarian cancer cells (22). To evaluate whether similar alterations in DNA repair may be a potential explanation for the enhanced effects of antireceptor antibody and radiation, we measured DNA repair induced by radiation in SKBR3 and MCF-7/HER-2 cells using autoradiographic localization of [³H]thymidine over cell nuclei (36) to provide a quantitative measure of this phenomenon. As expected, radiation exposure induces enhanced UDS in SKBR3 cells (Fig. 3). Exposure to rhuMAb HER-2 alone has no such effects on these cells. The radiation-induced effect, however, was blocked by pretreatment of the cells with antireceptor antibody (Fig. 3). To determine whether this phenomenon was restricted to a specific cell line and to study its association with HER-2 overexpression, we performed similar studies in MCF-7 and MCF-7/HER-2 cells. These two cell lines are identical to one another except for the presence of HER-2 overexpression in the MCF-7/HER-2 cells. Radiation elicited a marked increase in DNA repair in the PAR, CON, and HER-2 cells. However, this radiation-induced effect was blocked by rhuMAb HER-2 in the MCF-7/HER-2 cells specifically and did not occur in control cells. These data confirm that rhuMAb HER-2 interferes with DNA repair only in those cells overexpressing the HER-2 receptor (Fig. 3). Using an alternative measure of DNA repair, we observed the same phenomenon with a transfected CMV-driven β-galactosidase reporter plasmid (36). At 24 h posttransfection, the extent of repair was assayed by measuring reporter DNA expression in MCF-7/HER-2 cells that were either incubated with rhuMAb HER-2 or control media after transfection (Fig. 4). The transfected cells were then stained with 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside, a substrate for β-galactosidase, to distinguish β-galacto-sidase-positive and -negative cells. In the presence of substrate, cells expressing the reporter bacterial β-galactosidase protein appear blue and the percentage of stained cells can be quantitated. These data show that antibody treatment elicits blockade of repair of the radiation-damaged reporter DNA (Fig. 4), again demonstrating a therapeutic advantage seen in cells overexpressing HER-2 after treatment with a combination of antibodies to the HER-2 receptor and radiation.
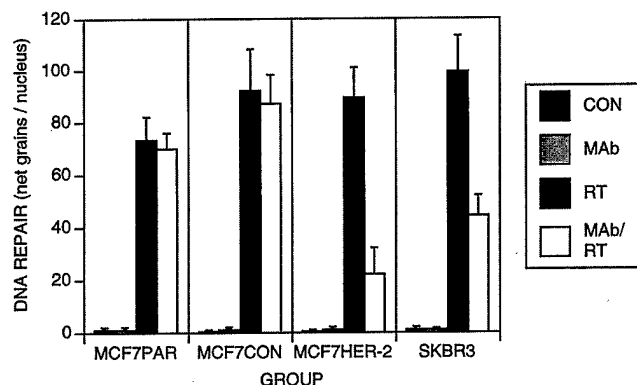

Fig. 3. Monoclonal antibody to HER-2 receptor blocks DNA repair (UDS) after radiation treatment of human breast cancer cells. Breast cancer cells (4×10^6 cells per dish) were plated for 24 h. Then, cells were incubated with medium containing 1% FCS and 20 mM hydroxyurea. MCF-7 parental (*MCF7PAR*), control (*MCF7CON*), and HER-2-overexpressing (*MCF7HER-2*) cells and SKBR3 breast cancer cells were treated with 2 Gy of radiation with or without HER-2 MAb at 200 μg/ml. Measurement of DNA repair by autoradiographic localization of radioactive thymidine was tabulated in MCF-7/PAR, MCF-7/CON, MCF-7/HER-2 cells, and SKBR3 cells by established methods. Counts of developed silver grains in the photographic emulsion overlying cell nuclei were compared after treatment with rhuMAb HER-2 (*MAb*), radiation (*RT*), rhuMAb HER-2/radiation (*MAb/RT*), or control (*CON*).
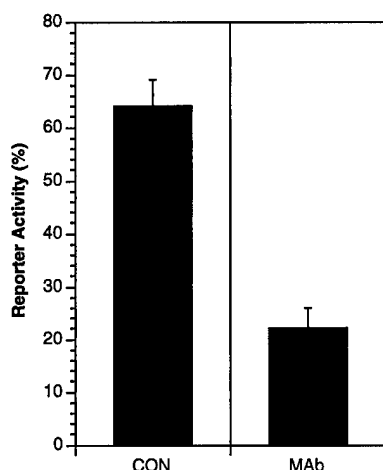
Fig. 4. HER-2 antireceptor antibody affects repair of radiation-damaged reporter DNA in human breast cancer cells. CMV-driven β-galactosidase reporter plasmid was exposed to radiation *in vitro* and then transfected into MCF-7/HER-2 cells. At 24 h after transfection was completed, we assayed the extent of repair by measuring reporter DNA expression in MCF-7/HER-2 cells that were incubated without antibody (*CON*) or with rhuMAb HER-2 (*MAb*) beginning at the end of the transfection. Reporter activity is presented as the percentage of blue-stained cells in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a substrate for β-galactosidase.

Influence of Antireceptor Antibodies on Cell Cycle Regulation in Human Breast Cancer Cells. To further characterize effects of rhuMAb HER-2 on breast cancer cell growth, analyses of cell cycle phase distribution were conducted. Previous reports have shown that the 4D5 antibody directed against the HER-2 receptor reduces the fraction of HER-2-overexpressing cells in S phase (34). Using a similar approach, we grew MCF-7/HER-2 cells in serum-depleted medium and then treated them with 1 or 10% serum-enriched medium. The cells were then exposed to 4 Gy of radiation with or without prior exposure to rhuMAb HER-2. Results of these studies demonstrate that maintenance of cells in serum-depleted medium reduces the fraction of cells in S-phase compared to control cells in medium with 10% serum (Fig. 5). Treatment with rhuMAb HER-2 reduces the fraction of MCF-7 HER-2 cells in S phase at both 24 and 48 h. Radiation treatment of the cells also promotes cell cycle arrest, predominantly at G_1, again resulting in a low S-phase fraction at 24 and 48 h. In the presence of rhuMAb HER-2, radiation elicits a similar reduction in S phase at 24 h; however, a significant reversal of the cell cycle arrest appears by 48 h postradiation ($P < 0.001$; Fig. 5). Unlike the non-antibody-treated cells, the S-phase fraction of these cells is significantly greater at 48 h compared to the fraction at 24 h. These results indicate that early escape from cell cycle arrest in the presence of antireceptor antibody may not allow sufficient time for completion of DNA repair in HER-2-overexpressing cells.

Influence of Antireceptor Antibodies on Regulation of p21WAF1 Transcript Levels, Protein Levels, and Phosphorylation State. To further explore the molecular basis for these observations, we performed a series of studies evaluating the p21WAF1 activity in these cells. The tumor suppressor gene *p53* is known to be a critical mediator of the cellular response to DNA damage (25, 27). Induction of the CDK inhibitor, p21WAF1, in response to DNA damage occurs primarily through a transcriptional mechanism that requires interaction of the p53 protein with a p53-binding site in the p21WAF1 promoter (28). Recent studies, however, have shown that induction of p21WAF1 following growth factor stimulation may not always require p53 and may, instead, be directly activated by MAP kinase (31). Consistent with this is the observation that withdrawal of growth factors *in vitro* is associated with down-regulation of p21WAF1 expression and with enhanced cell killing in response to

DNA damage (3). It is known that basal levels of p21WAF1 are not sufficient to cause cell cycle arrest and that failure of adequate p21WAF1 induction after DNA damage is associated with failure of cell cycle arrest. This results in a reduced time for completion of DNA repair. To assess the activity of p21WAF1 in response to DNA damage in MCF-7/HER-2 cells in the presence or absence of the anti-HER-2 antibody, we first performed Northern blot analyses of p21WAFI expression levels. MCF-7/HER-2 cells were treated with rhuMAb HER-2 alone or prior to radiation exposure to 6 Gy. Parallel cells were treated with either control solution alone or radiation alone. At 6, 12, and 24 h, cells were processed for RNA extraction and determination of p21WAF1 transcript levels. As expected, progressive induction of p21WAF1 transcripts was seen at 6–24 h postradiation treatment (Fig. 6A); however, increased levels of p21WAF1 transcript were not sustained in MCF-7 HER-2 cells that had been exposed to radiation in the presence of rhuMAb HER-2. Although p21WAF1 transcript level increases at 6–12 h, it is comparable to baseline levels by 24 h (Fig. 6A). Moreover, the level of p21WAF1 at 24 h is markedly less than the levels seen after radiation given without antibody. A clear reduction in the basal level of p21WAF1 also occurred after 12–24 h exposure to antibody alone, compared to controls. In contrast, the level of p53 transcripts was only slightly increased by radiation after 6 h or 24 h, and no attenuation of the transcript level occurred after combined treatment with rhuMAb HER-2 (Fig. 7A). An additional transcript, cyclin D1, showed no variation with antibody, radiation or combination therapy (Fig. 7B).

Western analyses of the level of p21WAF1 protein in MCF-7/HER-2 cells likewise show enhanced amounts of the protein at 6–24 h after radiation (Fig. 6B); however, consistent with the Northern blot studies, treatment of cells with antireceptor antibody elicits a reduced level of p21WAF1 protein under basal conditions and blunts the anticipated response to radiation therapy at 12–24 h, as compared to controls. Radiation with 6 or 10 Gy elicits a significant increase in the level of mdm2 protein, whereas a pronounced decrease in mdm2 protein level occurs when radiation is administered in the presence of anti-HER-2 receptor antibody (Fig. 8A). Similarly, the level of p53 protein increases in response to radiation after 6 and 24 h, and, as with p21WAF1, there is a slight reduction in the expected response to radiation when cells are treated concomitantly with rhuMAb HER-2
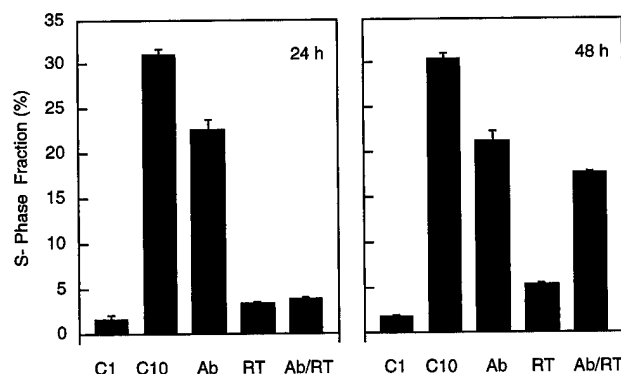

Fig. 5. Effects of antibody to HER-2 receptor and irradiation on cell cycle progression in HER-2-overexpressing human breast cancer cells. MCF-7 cells with HER-2 overexpression were plated and allowed to adhere. After 48 h, the monolayers were washed with PBS and incubated in medium with 1% DCC-stripped FCS to arrest cell growth. After 48 h, this medium was replaced with medium containing either 1% DCC-treated FCS (*C1*) or 10% FCS without (*C10*) or with 200 μg/ml antibody to HER-2 receptor (*Ab*). After 4 h, cells with 10% FCS but without antibody were treated with 4 Gy radiation (*RT*), and cells with 10% FCS plus 200 μg/ml antibody were likewise treated with 4 Gy radiation (*Ab/RT*). Cells from all treatment groups were incubated further for 24 or 48 h. Cells were then trypsinized, washed, fixed in ice-cold methanol, and prepared for determination of S-phase fraction by established methods, using DNA staining with propidium iodide and flow cytometry (34).
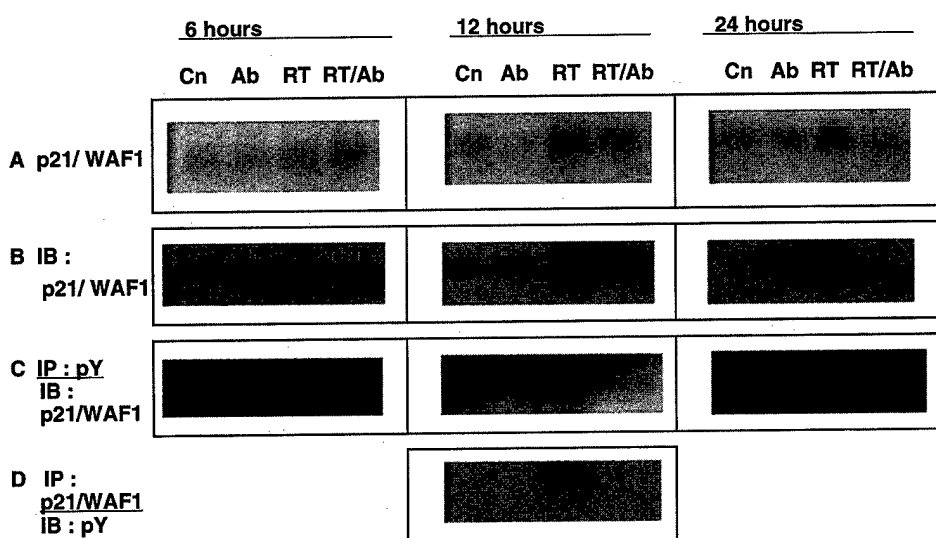
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Fig. 6. Monoclonal antibody to HER-2 receptor alters p21WAF1 transcript and protein levels and the tyrosine phosphorylation of p21WAF1 after radiation treatment of human breast cancer cells with HER-2 overexpression. *A*, MCF-7 HER-2 cells were treated with control solution (*Lane Cn*), 200 μg/ml rhuMAb HER-2 alone (*Lane Ab*), 6 Gy radiation alone (*Lane RT*), or 200 μg/ml rhuMAb HER-2 in combination with 6 Gy radiation (*Lane Ab/RT*). After 6, 12, or 24 h, cells were processed for preparation of RNA and determination of p21WAF1 transcripts using Northern blot. *B*, MCF-7 HER-2 cells were treated with control solution (*Lane Cn*), 200 μg/ml rhuMAb HER-2 alone (*Lane Ab*), 6 Gy radiation alone (*Lane RT*), or 200 μg/ml rhuMAb HER-2 in combination with 6 Gy radiation (*Lane Ab/RT*). After 6, 12, or 24 h, cells were processed for Western blot analysis and determination of p21WAF1 levels by immunoblot. *C*, MCF-7 HER-2 cells were treated with control solution (*Lane Cn*), 200 μg/ml rhuMAb HER-2 alone (*Lane Ab*), 6 Gy radiation alone (*Lane RT*), or 200 μg/ml rhuMAb HER-2 in combination with 6 Gy radiation (*Lane Ab/RT*). After 6, 12, or 24 h, cells were processed for Western blot analysis and determination of the level of tyrosine phosphorylation of p21WAF1 by first immunoprecipitating with antiphosphotyrosine antibody and then immunoblotting with anti-p21WAF1 antibody. *D*, MCF-7 HER-2 cells were treated with control solution (*Lane Cn*), 200 μg/ml rhuMAb HER-2 alone (*Lane Ab*), 6 Gy radiation alone (*Lane RT*), or 200 μg/ml rhuMAb HER-2 in combination with 6 Gy radiation (*Lane Ab/RT*). After 12 h, cells were processed for Western blot analysis and determination of the level of tyrosine phosphorylation of p21WAF1 by first immunoprecipitating with anti-p21WAF1 antibody and then immunoblotting with antiphosphotyrosine antibody. See text for additional details.

(Fig. 8*B*). In contrast, the level of PCNA and bcl-2 protein is unchanged at 6 and 24 h postradiation with or without rhuMAb HER-2 treatment (Fig. 8, *C* and *D*). These results are consistent with independent reports on depletion of p21WAF1 after withdrawal of growth factors (3) and suggest an important role for growth factor pathways in modulating the activity of some proteins that regulate the cell cycle in response to DNA damage.

In view of the crucial role of tyrosine phosphorylation in regulating the activity of diverse signaling molecules (4, 8, 9), we assessed the potential influence of the HER-2 receptor pathway on phosphorylation of tyrosine residues in p21WAF1 (28). We first tested whether p21WAF1 is a substrate for tyrosine phosphorylation induced by radiation. A number of protein kinases are known to be induced by

stress and/or DNA damage and are hypothesized to play a role in DNA repair by phosphorylating regulatory proteins (30). MCF-7/HER-2 cells show little to no tyrosine phosphorylation of p21WAF1 under basal conditions (Fig. 6*C*); however, radiation exposure elicits p21WAF1 tyrosine phosphorylation, which is evident after 6 and 12 h and dissipates by 24 h (Fig. 6*C*). In the presence of anti-HER-2 receptor antibody, radiation-induced tyrosine phosphorylation of p21WAF1 occurs by 6 h, but the phosphorylation is not sustained and returns to baseline levels by 12 h (Fig. 6, *C* and *D*). These results suggest that dysregulation of radiation-induced p21WAF1 tyrosine phosphorylation occurs after treatment with antireceptor antibody, and this event may adversely influence the cell response to DNA damage.

DISCUSSION

The potential role of *HER-2* and related *erb B* proto-oncogenes in the modulation of sensitivity to radiation has been suggested from some studies (20, 21). Transformation of NIH-3T3 cells with HER-2 cDNA from esophageal carcinoma leads to induction of radiation resistance (20). Here, we used matched MCF-7 parent and daughter human breast cancer cells, which differ in their HER-2 expression level, to evaluate effects on radiation sensitivity. These paired cells were used to circumvent the possibility that phenomena due to effects other than HER-2 overexpression (*i.e.*, cell line specific), might be observed. In addition, we used the naturally overexpressing cell line SKBR3 to ensure that the results were not restricted to a single cell line and were not due solely to artificial engineering of the cells. Using this approach, we were able to directly compare *in vitro* radiation sensitivities of parental cells with low-expression of HER-2 to identical daughter cells with high-expression of HER-2. The data demonstrate that the D_{10} of HER-2-overexpressing breast cancer cells is increased by ~25% compared to cells with the normal complement of HER-2 receptors. These findings are consistent with independent
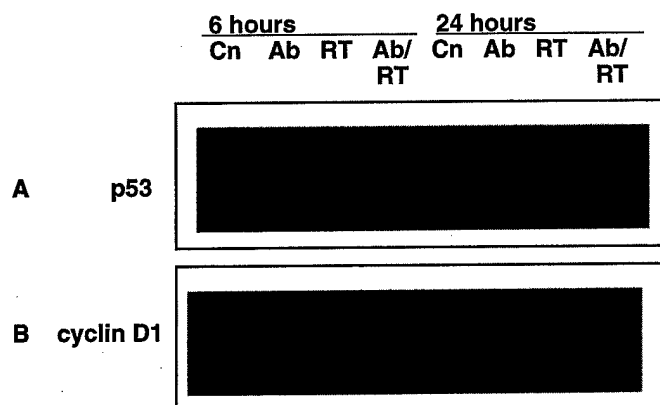

Fig. 7. Effects of monoclonal antibody to HER-2 receptor on p53 and cyclin D transcripts after radiation treatment of human breast cancer cells with HER-2 overexpression. MCF-7/HER-2 cells were treated with control solution (*Lane Cn*), 200 μg/ml rhuMAb HER-2 alone (*Lane Ab*), 6 Gy radiation alone (*Lane RT*), or 200 μg/ml rhuMAb HER-2 in combination with 6 Gy radiation (*Lane Ab/RT*). After 6 or 24 h, cells were processed for preparation of RNA and determination of p53 (*A*) and cyclin D (*B*) transcripts using Northern blot.
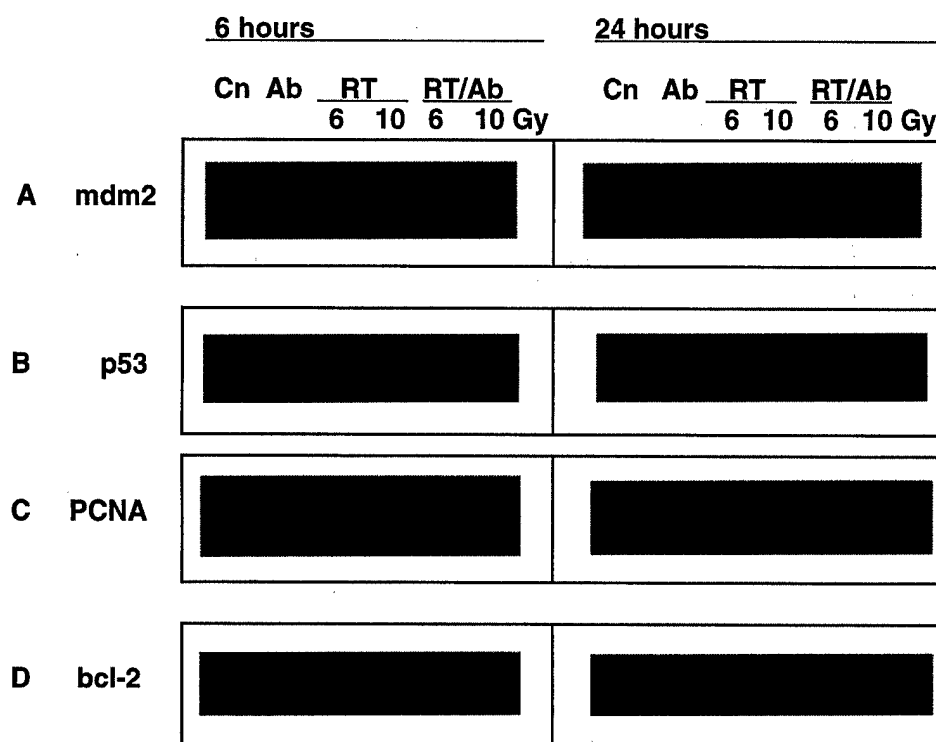
Fig. 8. Effects of monoclonal antibody to HER-2 receptor on protein levels of mdm2, p53, PCNA, and bcl-2 after radiation treatment of human breast cancer cells with HER-2 overexpression. MCF-7/HER-2 cells were treated with control solution (*Lane Cn*), 200 µg/ml rhuMAb HER-2 alone (*Lane Ab*), 6 or 10 Gy radiation alone (*Lanes RT, 6* and *10*, respectively), or 200 µg/ml rhuMAb HER-2 in combination with 6 Gy or 10 Gy radiation (*Lanes Ab/RT, 6* and *10*, respectively). After 6 or 24 h, cells were processed for Western blot analysis, and levels of mdm2 (*A*), p53 (*B*), PCNA (*C*), and bcl-2 (*D*) were determined. See text for additional details.

studies in which antisense oligonucleotides directed against the HER-2 gene were able to reverse the radiation resistance of human tumor cell lines with HER-2 overexpression (42). These analyses suggest a potential biological role for the *HER-2* oncogene in resistance to radiation treatment and may have important implications in the clinical management of patients whose breast cancers contain this alteration. To evaluate the therapeutic advantage of combined treatment with radiation and antibodies to HER-2, we conducted a series of *in vitro* studies, which show that anti-HER-2 receptor antibody enhances radiation-induced killing of naturally overexpressing SKBR3 human breast cancer cells as well as MCF-7 cells engineered to overexpress this receptor. Further tests demonstrate a significant growth-inhibitory therapeutic advantage of ionizing radiation combined with rhuMAb HER-2 in HER-2 overexpressing human breast cancer xenografts in nude mice. Mice receiving radiation alone do not have significantly reduced tumor volumes over the 7-week observation period, and no tumor remissions were observed. Treatment with rhuMAb HER-2 alone similarly elicited no significant tumor remissions. In contrast, the combination of radiation and antibody therapy produced marked reductions in tumor growth, and all animals receiving both rhuMAb HER-2 and radiation show complete tumor remissions, demonstrating a marked *in vivo* enhancement of radiation efficacy when given with anti-HER-2 antibody in HER-2-overexpressing breast cancers.

A spectrum of lesions is known to be induced in DNA by radiation, and DNA repair plays an important role in the recovery of cells from the toxicity of radiation exposure (41). Changes in DNA repair have been reported to occur in HER-2-overexpressing cells after treatment with antibodies to HER-2 receptor (22). To further evaluate the possible role of DNA repair as an explanation for the therapeutic advantage of anti-HER-2 antibody combined with DNA-damaging radiation, we measured DNA repair, cell cycle regulation and selected molecular alterations induced by radiation in breast cancer. These studies show that radiation enhances UDS, a measure of DNA repair, in human breast cancer cells with HER-2 overexpression. This radi-

ation-induced effect is blocked by treatment of the cells with anti-HER-2 antibody. Additional tests of DNA repair using a CMV-driven β-galactosidase reporter plasmid exposed to radiation *in vitro* demonstrate that repair of radiation-damaged DNA proceeds in the absence of rhuMAb HER-2 but is significantly reduced when the antibody is administered to human breast cancer cells containing the HER-2 alteration. Moreover, this phenomenon is specific to cells overexpressing HER-2.

Prior studies have shown that the 4D5 antibody reduces the fraction of HER-2-overexpressing cells in S phase (33). Conducting additional studies of cell cycle phase distribution here, we demonstrated that rhuMAb HER-2 reduced the fraction of MCF-7 HER-2 cells in S phase at both 24 and 48 h (33). This study also indicates that radiation promotes cell cycle arrest predominantly at G_1, with a low S-phase fraction observed at 24 and 48 h. In the presence of rhuMAb HER-2, radiation elicited a similar reduction in S-phase at the early time point, *i.e.*, 24 h, but a significant reversal of cell cycle arrest occurred at 48 h postradiation exposure. Hence, early escape from cell cycle arrest in the presence of anti-HER-2 antibody may not allow sufficient time for completion of DNA repair in HER-2-overexpressing cells, resulting in accumulation of DNA damage and greater cell death.

The tumor suppressor gene product p53 is a critical mediator of the cellular response to DNA damage. Although induction of the CDK inhibitor, p21WAF1, in response to DNA damage occurs primarily through a transcriptional mechanism involving p53 (28), some reports suggest that p21WAF1 may enhance the radiosensitivity of tumor cells independent of p53 (31, 43). In p21WAF1 −/− colon cancer cells, p21WAF1 deficiency is associated with a prominent defect in DNA repair (37). Recent work suggests that certain growth factors interacting with their respective receptors may provide an alternate pathway for regulation of p21WAF1 expression. Activation of some growth factor stimulatory pathways results in induction of p21WAF1, which does not require p53 and may, instead, be activated by MAP kinase (31). In addition, withdrawal of growth factors *in vitro* has been associated with down-regulation of p21WAF1 expression and

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enhanced cell killing in response to DNA damage. This study provides further evidence that the growth factor receptor, HER-2, can modulate DNA damage response pathways in human breast cancer cells and suggests that this cross-communication may involve modulation of p21WAF1. A notable reduction in the basal level of p21WAF1 occurred after exposure to anti-HER-2 receptor antibody when compared with controls, indicating that interactions with the HER-2 pathway can directly affect p21WAF1 expression. These results and independent reports on depletion of p21WAF1 after withdrawal of growth factors (3, 44) suggest an important role for at least some growth factor receptor pathways in modulating the activity of proteins that regulate the cell cycle in response to DNA damage. It is well known that basal levels of p21WAF1 are not sufficient to cause cell cycle arrest and that failure of adequate p21WAF1 induction after DNA damage is associated with failure of cell cycle arrest, resulting in reduced time for completion of DNA repair. After radiation, the expression of p21WAF1 is increased in MCF-7/HER-2 cells, but the increased levels of p21WAF1 transcripts and protein are not sustained in HER-2-overexpressing cells exposed to radiation in the presence of rhuMAb HER-2. In contrast, levels of p53 transcript and protein were only slightly increased by radiation, and only minimal reductions occurred on combined treatment with rhuMAb HER-2. This suggests that induction of p21WAF1 in HER-2-overexpressing MCF-7 cells may be less dependent on regulation by p53 and may involve alternative signal transduction pathways (31, 42, 44).

In view of the crucial role of phosphorylation in regulating the activity of diverse signaling molecules (4, 8, 9), we assessed the potential influence of the HER-2 receptor signaling pathway and radiation on phosphorylation of tyrosine residues in p21WAF1 (28). A number of protein kinases are well known to be induced by stress and/or DNA damage and are hypothesized to play a role in DNA repair by phosphorylation of regulatory proteins (30). Although MCF-7/HER-2 cells show little to no tyrosine phosphorylation of p21WAF1 under basal conditions, radiation treatment induces a transient tyrosine phosphorylation of p21WAF1, an effect that is clearly diminished by anti-HER-2 receptor antibody. These results suggest that dysregulation of radiation-induced p21WAF1 tyrosine phosphorylation occurs after treatment with antireceptor antibody. The potential outcome of this molecular alteration on the biological activity of p21WAF1 remains to be determined. However, independent studies show that DNA damage promotes specific alterations in the phosphorylation state of other DNA-regulatory factors, such as p53 (45) and BRCA1 (46), leading to changes in nuclear localization and in specific molecular interactions. Tyrosine residues of p21WAF1 occur in functional domains known to be associated with nuclear localization and with binding to cyclin/CDKs and to PCNA (28, 47). Moreover, the tyrosine residues in p21WAF1 of human and mouse origin are highly conserved in corresponding regions of other human CDK inhibitors, p27^{Kip1} and p57^{Kip2} (47), suggesting that these proteins may share a similar mechanism of action. It will be important to direct future studies to investigation of the role of tyrosine phosphorylation of p21WAF1 in critical molecular interactions.

Significant data support the hypothesis that p21WAF1 may play a vital role in mediating the rhuMAb HER-2 effects on DNA damage pathways in HER-2-overexpressing breast cancer cells. DNA replication and repair may be coordinated by differential effects of p21WAF1 on replicative and repair DNA synthesis. The p21WAF1 protein interacts with CDKs and PCNA, a protein important for regulation of both DNA replication and repair processes (3, 31, 48). This work suggests that alterations in other regulatory proteins, such as mdm2 (29), may also contribute to this process. Radiation elicits a significant rise in the level of mdm2 protein that is sustained over several hours. However, when radiation is administered in the pres-

ence of anti-HER-2 receptor antibody, the initial increment in the level of mdm2 protein is not maintained, with a pronounced decline in mdm2 by 24 h. These changes in mdm2 levels may be important because the oncoprotein contains inhibitory domains that can interfere with both p53-dependent (49) and -independent (50) transcriptional activity. Promotion of apoptosis (3) by rhuMAb HER-2 could be another postulated outcome of treatment, but we have been unable to document alterations in bcl-2 protein or DNA fragmentation in MCF-7/HER-2 cells after low, sublethal doses of ionizing radiation with or without anti-HER-2 antibody (51). Nevertheless, the available evidence suggests that pathways of DNA replication, DNA repair and DNA degradation may have common regulatory elements, with the final cellular outcome being dependent on the extent of DNA damage (48).

Future work will be required to fully understand how a MAP kinase may play a role in the regulation of p21WAF1 through growth factor receptors. Heregulin, a natural ligand to HER-2/HER-3 heterodimers, induces transient phosphorylation of HER-2 protein, promoting downstream activation of MAP kinase (38, 52). In contrast, antibodies to the HER-2 receptor induce prolonged phosphorylation and downregulation of HER-2 protein and disrupt the association of HER-2 with HER-3 (38, 52–54). Similar to anti-HER-2 antibodies, tyrosine kinase inhibitors with specificity for the HER-2 kinase are also known to enhance the sensitivity of HER-2-overexpressing cancer cells to DNA-damaging agents (55). Although the activity of the anti-HER-2 antibody remains to be fully characterized, downstream effects of HER-2 stimulation, such as activation of MAP kinase and other protein kinases, are likely to be affected by rhuMAb HER-2.

This study suggests that human breast cancer cells damaged by radiation may be especially vulnerable to injury if they are also deprived of essential signal transduction mechanisms by disruption of the HER-2 growth factor receptor pathway. Growth factor receptors appear to play a significant role in the regulation of cell cycle checkpoints and repair of DNA damage, and manipulation of this pathway in the clinic using rhuMAb HER-2 may provide therapeutic benefit to patients with HER-2-overexpressing breast cancers.

ACKNOWLEDGMENTS

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